# LUCIFERASE EXPRESSION ALLOWS BIOLUMINESCENCE IMAGING BUT IMPOSES LIMITATIONS ON THE ORTHOTOPIC MOUSE (4T1) MODEL OF BREAST CANCER

**Baklaushev VP[1,2]*, Kilpeläinen A[1,3], Petkov S[3], Abakumov MA[1], Grinenko NF[1], Yusubalieva GM[4], Latanova A A[5,6], Gubskiy IL[1], Zabozlaev FG[2], Starodubova ES[5,6], Abakumova TO[4], Isaguliants MG[3,6,7,8]*#, Chekhonin VP[1,4#]**

[1]Research and Education Center for Medical Nanobiotechnology, Pirogov Russian National Research Medical University, Ministry of Health of the Russian Federation; [2]Federal Research and Clinical Center of Specialized Medical Care and Medical Technologies, Federal Biomedical Agency of the Russian Federation, Moscow, Russia; [3]Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden; [4]Department of Fundamental and Applied Neurobiology, Serbsky National Research Center for Social and Forensic Psychiatry, Ministry of Health of the Russian Federation, Moscow, Russia; [5]Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia; [6] Chumakov Federal Scientific Center for Research and Development of Immunobiological Preparations, Moscow, Russia; [7] N.F. Gamaleya Research Center of Epidemiology and Microbiology, Moscow, Russia; [8]Riga Stradins University, Riga, Latvia.

**Corresponding authors:*

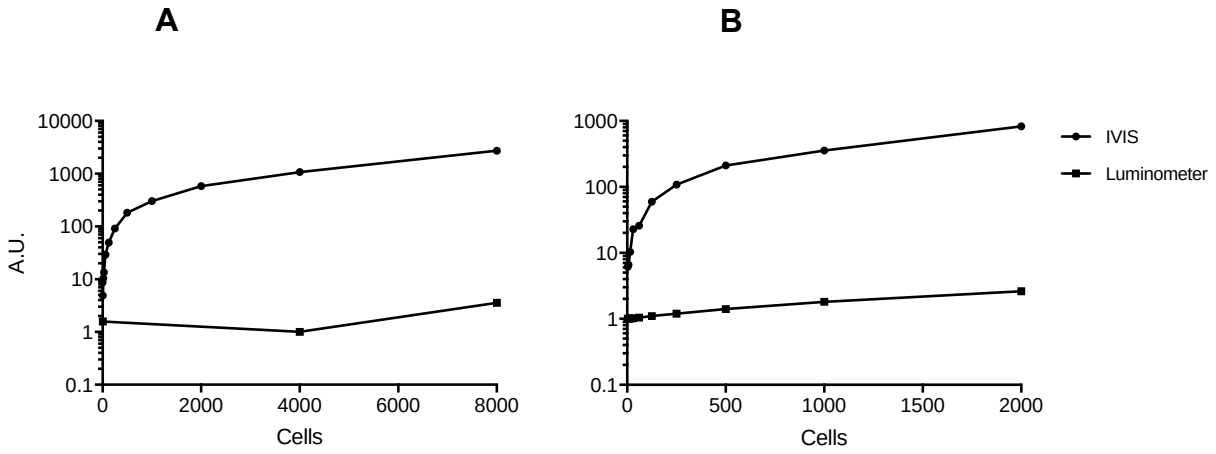
Vladimir Baklaushev, MD,PhD, Pirogov Russian National Research Medical University, and Federal Research and Clinical Center of Specialized Medical Care and Medical Technologies, 28 Orekhovyi bulvar, 115682 Moscow, Russia; serpoff@gmail.com;

Maria Isaguliants, Associate Prof, PhD; N.F. Gamaleya Research Center of Epidemiology and Microbiology and Chumakov Federal Scientific Center for Research and Development of Immunobiological Preparations, 123098 Moscow, Russia; and Department of Research, Riga Stradins University, Dzirciema iela 16, LV-1007, Riga, Latvia maria.issagouliantis@rsu.lv

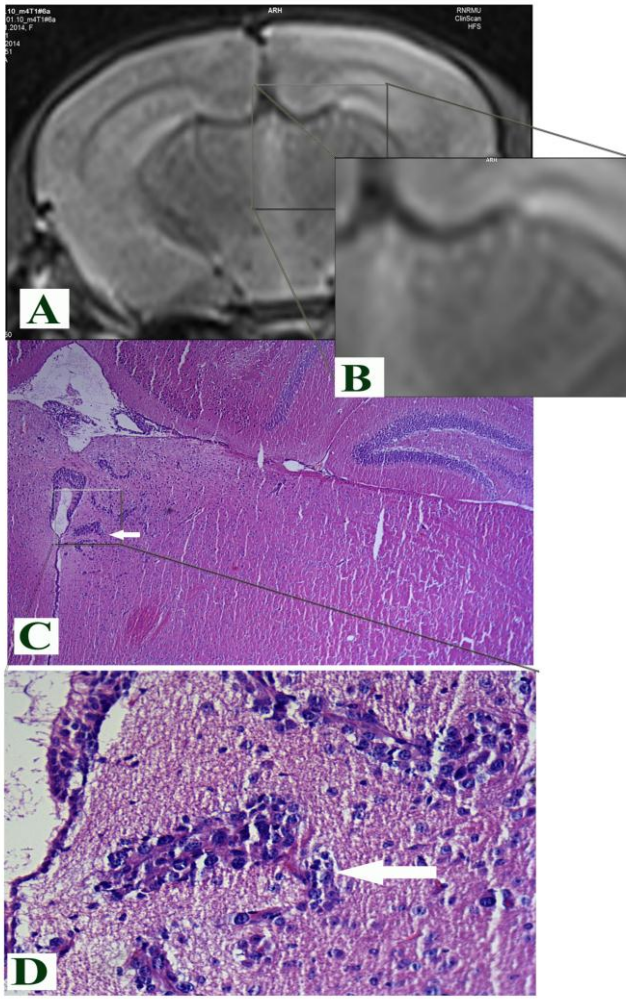
[#] *Shared last authorship.*

| Minutes | Discovery? | P value | Mean1 | Mean2 | Difference | SE of difference | t ratio | df | q value |
|---|---|---|---|---|---|---|---|---|---|
| 75 | No | 0.277116 | 21348 | 19600 | 1748 | 1521 | 1.149 | 10 | 0.391842 |
| 255 | No | 0.26257 | 24217 | 22698 | 1519 | 1280 | 1.187 | 10 | 0.391842 |
| 435 | No | 0.978091 | 26922 | 26945 | -23.04 | 818.3 | 0.02816 | 10 | 0.942968 |
| 615 | No | 0.095755 | 31038 | 29883 | 1155 | 628.1 | 1.839 | 10 | 0.169247 |
| 795 | Yes | 0.00016 | 35726 | 30633 | 5092 | 869.5 | 5.856 | 10 | 0.003398 |
| 975 | No | 0.037506 | 37606 | 35485 | 2121 | 884.8 | 2.397 | 10 | 0.08839 |
| 1155 | No | 0.01838 | 43288 | 39580 | 3708 | 1318 | 2.813 | 10 | 0.064973 |
| 1335 | No | 0.2974 | 44133 | 45100 | -967.3 | 879.9 | 1.099 | 10 | 0.394241 |
| 1515 | No | 0.003182 | 45877 | 48669 | -2792 | 724 | 3.856 | 10 | 0.022493 |
| 1695 | No | 0.779784 | 50133 | 49804 | 328.5 | 1144 | 0.2873 | 10 | 0.787582 |
| 1875 | No | 0.02942 | 62512 | 55869 | 6643 | 2616 | 2.539 | 10 | 0.077999 |
| 2055 | No | 0.004731 | 72895 | 66825 | 6069 | 1679 | 3.615 | 10 | 0.025088 |
| 2235 | No | 0.43477 | 84189 | 81954 | 2235 | 2747 | 0.8137 | 10 | 0.512304 |
| 2415 | No | 0.021903 | 89841 | 94537 | -4696 | 1732 | 2.711 | 10 | 0.066368 |
| 2595 | No | 0.001059 | 100725 | 111050 | -10325 | 2269 | 4.55 | 10 | 0.011229 |
| 2775 | No | 0.517941 | 122691 | 125580 | -2889 | 4311 | 0.6701 | 10 | 0.578185 |
| 2955 | No | 0.658367 | 148107 | 145954 | 2153 | 4725 | 0.4557 | 10 | 0.698198 |
| 3135 | No | 0.375986 | 170646 | 167147 | 3499 | 3777 | 0.9265 | 10 | 0.469097 |
| 3315 | No | 0.092055 | 182451 | 190127 | -7677 | 4120 | 1.863 | 10 | 0.169247 |
| 3495 | No | 0.141079 | 213559 | 207145 | 6414 | 4013 | 1.598 | 10 | 0.230175 |
| 3675 | No | 0.077327 | 239627 | 229007 | 10620 | 5395 | 1.969 | 10 | 0.16401 |
| 3855 | No | 0.013446 | 278560 | 254667 | 23894 | 7977 | 2.996 | 10 | 0.05704 |

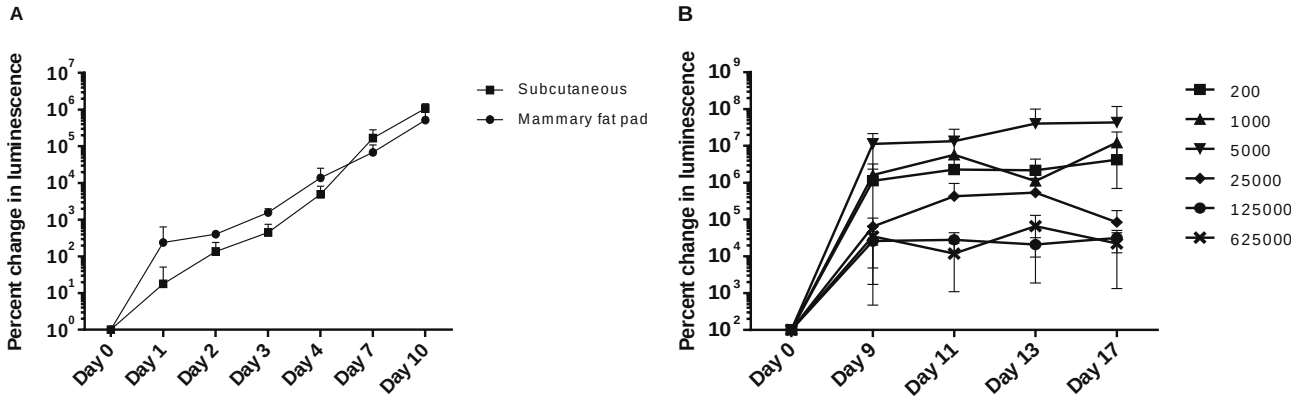
**Supplementary Table. 1. Results of** multiple t tests results comparing growth curves of 4T1Luc2 and 4T1Luc2D6 cell lines *in vitro* at all 22 time points. Statistical significance was determined using the Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with Q = 1%. Each test was analyzed individually, without assuming a consistent SD.



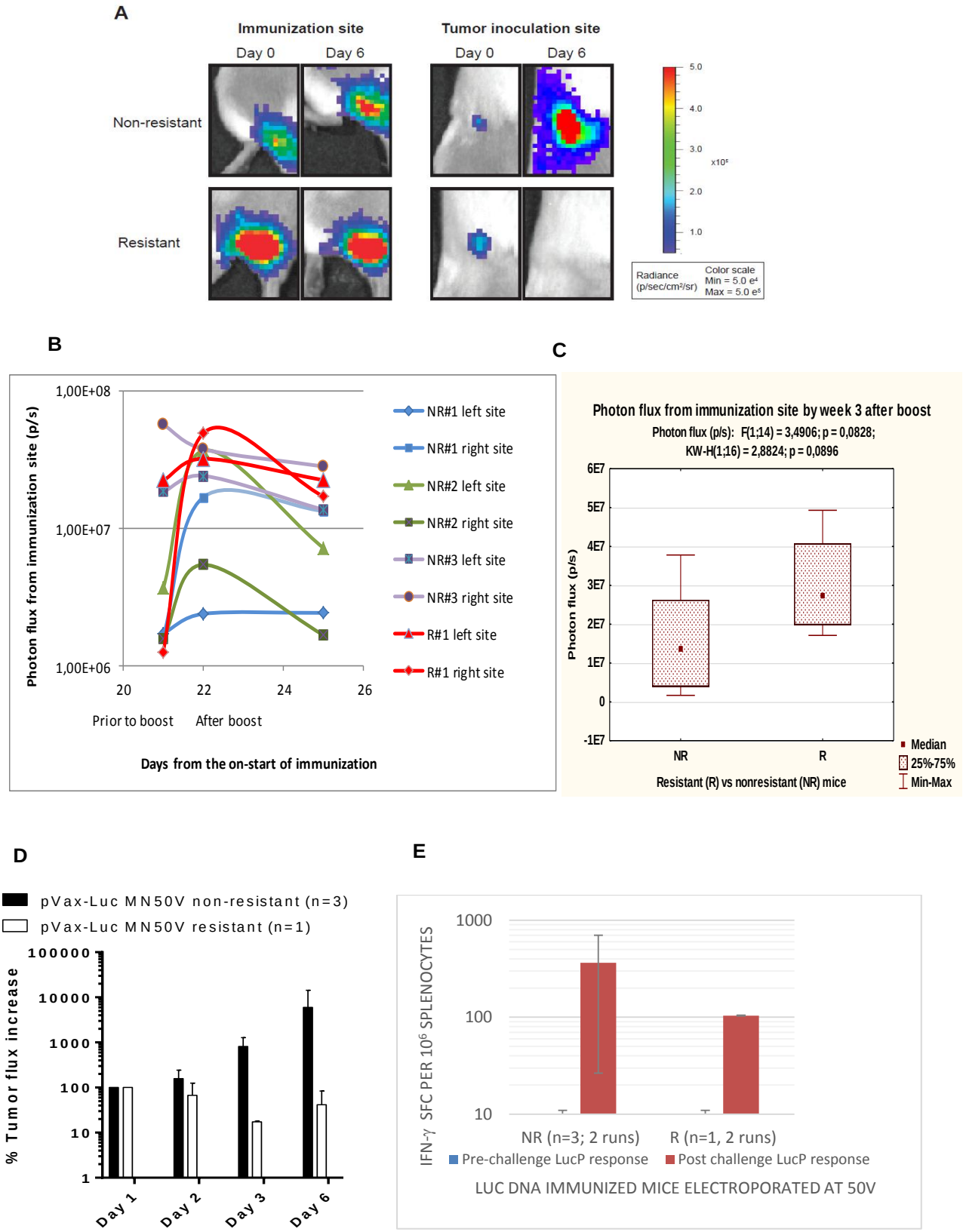

**Supplementary Fig. 1.** Correlation between the luminescence signal of 4T1lucD6 (A) and 4T1luc2 (B) cells registered by Spectrum CT (IVIS) and by Enspire devices (Luminometer). Pearson correlation coefficient, *r* between A.U. (photons/s) and cells was equal to 0.84 for 4T1lucD6, and 0.99 for 4T1luc2 cell lines.




**Supplementary Fig. 2** Visualization of brain metastases in mice implanted with 4T1 cells. Coronary projections by T2-weighted MRI image of the brain of a 4T1-implanted mouse 14 days after removal of the primary focus (A); magnified fragment of the MRI image, no metastases are clearly detectable (B); small perivasal metastases (less than 100 μm in diameter; indicated by arrow) in the paraventricular region of the thalamus, detected on a paraffin slice of the brain stained with hematoxylin–eosin (magnification 50×) (C); magnified fragment of brain slice, perivasal metastases indicated by arrow (magnification 200×) (D).

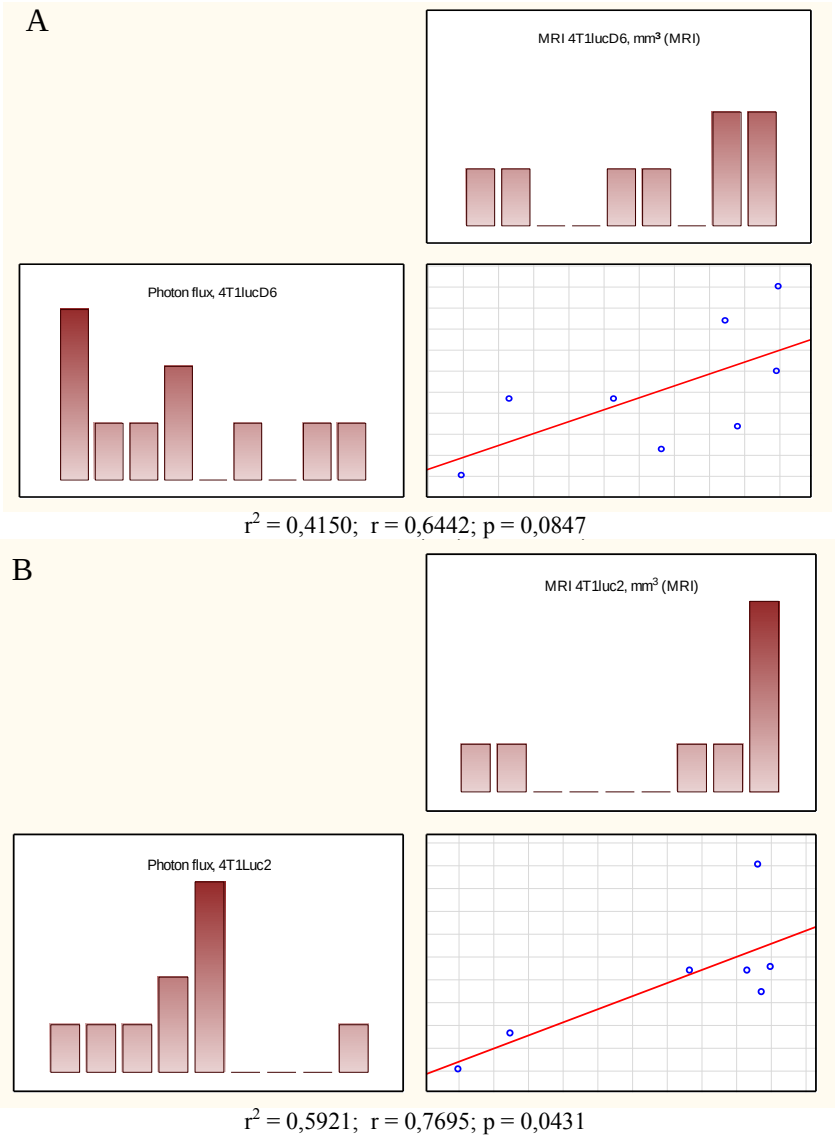



**Supplementary Fig. 3** Conditions for implantation of 4T1luc2 cells allowing reliable early detection of the effects of Luc DNA immunization on the initiation and growth of the primary focus. Orthotopically and ectopically implanted 4T1luc2 cells exhibit similar growth rate of the primary focus (A); Ectopic implantation of 5000 4T1luc2 cells per site results in the highest growth rate and the highest viability of the cells in the primary focus (B). Site of implantation and the number of implanted cells is depicted on the right. Each point demonstrates the results of two to four independent observations.




**Supplementary Fig. 4** Initiation of 4T1luc2 adenocarcinomas in BALB/c mice DNA-immunized with Luc DNA by intradermal injections followed by low voltage electroporation. BALB/c mice were immunized with Luc DNA followed by electroporation with a BEX device with driving pulses of 50V. Two weeks post boost immunization, mice were challenged with 5 x $10^3$ 4T1luc2 cells. BLI was performed directly after and on days 1, 2, 3 and 6 post the implantation. Sites of immunization with Luc DNA (left) and of implantation of 4T1luc2 cells (right) in immunized mice resistant and not resistant to tumor initiation at days 0 and 6 post the implantation (A); Total flux from

immunization sites prior to and post Luc DNA boost in mice resistant (R) and nonresistant (NR) to tumor challenge in photons/sec (B) with statistical evaluation (C); Luminescent signal from 4T1luc2 implantation sites visualized as percent change of the total flux compared to the level assessed directly after the implantation ± STDV (D); *In vitro* IFN-γ response of PBMC or splenocytes of Luc DNA mice to stimulation with peptide GFQSMYTFV representing the immunodominant CTL epitope of luciferase (LucP) before and after 4T1luc2 cell challenge± STDV (E).




**Supplementary Fig. 5** Correlation of tumor size evaluated by MRI ($mm^3$) and by BLI (photon flux) for tumors generated by 4T1lucD6 (A; R=0,6442, p=0,085) and 4T1Luc2 cell clones (B; R=0,77,p=0,043).